Journal of Medical Virology 59:415–423 (1999)

Hepatitis B Virus Core Promoter Mutations in Children With Multiple Anti-HBe/HBeAg Reactivations Result in Enhanced Promoter Activity

Patrick Gerner,[1] Ekkehart Lausch,[2] Michael Friedt,[3] Robert Tratzmüller,[4] Christian Spangenberg,[2] and Stefan Wirth[1]*

[1]*Children's Hospital Wuppertal, Cooperative Clinic of the University of Witten/Herdecke, Wuppertal, Germany*
[2]*Children's Hospital University of Mainz, Mainz, Germany*
[3]*Children's Hospital University of Hamburg, Hamburg, Germany*
[4]*Children's Hospital Augsburg, Augsburg, Germany*

Sera of two children were examined to determine whether specific hepatitis B virus (HBV) mutants may contribute to anti-hepatitis B e/hepatitis B e antigen (anti-HBe/HBeAg) reactivations during the course of chronic hepatitis B. The full-length HBV genome isolated from sera of patient 1 and the basic core promoter (BCP) from patient 2 were amplified and sequenced before and after several reactivations. The functional significance of the mutant BCP from patient 1 was studied using the luciferase assay. In both patients, rare mutations were found in the BCP at nucleotides $1764_{G\rightarrow T}/1766_{C\rightarrow G}$ and $1766_{C\rightarrow T}/1768_{T\rightarrow A}$ in case 1 and 2, respectively. In the BCP from patient 1, a putative new binding site for the transcription factor hepatocyte nuclear factor 3 (HNF3) was generated. The functional analyses of the mutant showed a 2.8-fold increase of core promoter activity, whereas the BCP variant of patient 2 was also identified to result in enhanced promoter activity. The alignment of full-length genomes from child 1 to the reference sequence showed 61 nucleotide substitutions. Furthermore, the time of reactivations from child 1 was always accompanied by selection of a precore mutation at nucleotide position 1899. In liver tissue of patient 1 before development of hepatocellular carcinoma only free viral sequences were found, whereas a single site integration of HBV was detected in hepatocytes after activation of carcinogenesis. Specific mutations in the HBV BCP of the two patients that are rarely present in chronic carriers were identified to increase the core promoter activity possibly by altering transcription factor binding, suggesting that these variants may be involved in the pathogenesis of frequent HBV reactivations. ***J. Med. Virol. 59:415–423, 1999.***

KEY WORDS: full-length; basic core promoter; transcription factor; infant; HCC; viral integration

INTRODUCTION

Due to their crucial role in the viral life cycle, specific mutations within the core promoter have been suggested to influence the clinical outcome of hepatitis B virus (HBV) infection. An important element of this promoter is the basic core promoter (BCP), located between nucleotides 1744 and 1804, which overlaps with the HBx open reading frame (ORF). A small region has been shown to represent a hot spot of mutations in patients with chronic hepatitis [Laskus et al., 1994; Okamoto et al., 1994; Kurosaki et al., 1996; Lindh et al., 1998] or fulminant hepatitis [Hasegawa et al., 1994; Sterneck et al., 1997, 1998; Inoue et al., 1998; Friedt et al., 1999]. It was also demonstrated that this particular region (nt 1749–1773) can be bound specifically by a number of transcription factors [Yu and Mertz, 1997]. Functional studies of mutations within this region revealed enhanced core promoter activity, more replication competent phenotypes, increased expression of Core protein/pregenomic RNA, and decreased hepatitis B e antigen (HBeAg) synthesis

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: WI 991/3-1.

*Correspondence to: Professor Dr. Stefan Wirth, Children's Hospital Wuppertal, Heusnerstr. 40, D-42283 Wuppertal, Germany. E-mail: wirth@klinikum-wuppertal.de

Accepted 28 May 1999

[Baumert et al., 1996; Buckwold et al., 1996; Günther et al., 1996].

To date, the genetic reasons for HBV reactivation, defined as reconversion from anti-hepatitis B e (anti-HBe) to HBeAg and a viral flare-up, have only been searched for in the preCore/Core open reading frame (ORF) of the HBV [Raimondo et al., 1990; Takeda et al., 1990; Loriot et al., 1995]. Nevertheless, there are several reports of conflicting results about the role of pre-C stop codon mutants for HBV reactivation.

To address this issue and to obtain more comprehensive insight into the sequence variability of the entire viral genome and the possible triggers for viral reactivation, HBV strains were studied from two patients on a sequential and functional basis. The two patients investigated in this study were an interesting in vivo model. First, the relatively short duration of the infection in the two children, aged 3 and 13 years, diminishes the number of naturally occurring nucleotide substitutions and may thus argue for the biological significance of the identified mutations. Second, both patients went through multiple reactivations. Third, follow-up sera were available immediately before and after the reactivations.

METHODS

Patients

Sera of two children with histologically confirmed chronic active hepatitis B (CAH) were collected from different stages of the disease with a follow up of 5 years (patient 1) and 6 years (patient 2), respectively (laboratory data are shown in Fig. 1). Both patients had repeated anti-HBe/HBeAg+ reactivations with a marked increase of HBV DNA and alanine aminotransferase (ALT) serum levels.

Patient 1, a 3-year-old boy of Turkish origin, was infected perinatally by his mother. He was treated with four courses of alpha-interferon (IFN) due to the HBeAg-positive reactivations and histologically confirmed progressive liver inflammation. The peak levels of ALT (761 IU/L) and of HBV DNA in serum (3650 pg/ml) were very high. Each treatment was followed by a marked decrease of transaminases and HBV DNA. Despite seroconversion to anti-HBe after the second, third, and during the fourth IFN therapy he developed HBV reactivation after each treatment. After the last treatment with IFN he remained anti-HBe seropositive. When the boy had been 2 years of age, liver histology had revealed cirrhosis; 6 years later, a hepatocellular carcinoma (HCC) was diagnosed. Twelve different serum samples were obtained for amplification and sequencing of full-length HBV genomes and of the BCP (Fig. 1).

Patient 2, a 10-year-old boy of Greek origin, was also infected by his mother with HBV at birth. After seroconversion to anti-HBe he experienced reactivation of hepatitis. Each of two courses of IFN treatment was again followed by seroconversion to e antibodies, whereas viral elimination was attained only after the second course of treatment. The BCP of four sera from phases before and after the reactivations were analyzed (Fig. 1, arrows).

Serological Markers

Hepatitis B surface antigen (HBsAg), HBeAg, anti-HBe, anti-HBs, and anti-HBc were assayed using commercial radioimmunoassays (Abbott Laboratories, North Chicago, IL). Both patients were negative for antibodies to hepatitis C virus, hepatitis D virus, and human immunodeficiency virus. HBV DNA was determined quantitatively by a liquid hybridization assay (Hybride Capture Systems, Digene Diagnostics, Beltsville, MD).

Polymerase Chain Reaction (PCR) Amplification, Subcloning, and Sequencing

An aliquot of 200 μl of each patient's serum was obtained to isolate viral DNA using the QIAamp Blood Kit (Qiagen, Chatsworth, CA). The DNA was eluted with 50 μl distilled water according to the manufacturer's recommendations. The HBV DNA isolated from 20 μl of serum was amplified by PCR in 50 μl of buffer containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM deoxynucleoside triphosphates (dNTPs), 1 U of Taq Polymerase (Boehringer Mannheim, Germany), and 30 pmol of primers P1–P8. The PCR was performed for 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The PCR products were visualized after electrophoresis in a 1.5% agarose gel and ethidium bromide staining. To minimize artificial mutagenesis for samples not detectable after the first amplification, a second PCR was carried out with 30 cycles and 1 U Taq DNA-Pwo DNA polymerase (Expand High Fidelity assay; Boehringer Mannheim) was used. In contrast to the Taq Enzyme, the Pwo Polymerase has a proofreading activity. An aliquot (40 μl) of the PCR product from one serum sample of patient 1 was ligated in *ECO RV*-digested plasmid Bluescript (Stratagene), transformed in *Escherichia coli* DH5alpha (BRL) and cloned.

Primers for amplification of four HBV fragments:

Fragment 1: P1–P2:
P1: 5′ TTT TTC ACC TCT GCC TAA TCA (1821–1841)
P2: 5′ TTG GGA TTG AAG TCC CAA TCT GG (2957–2935)
Fragment 2: P3–P4
P3: 5′ GGG TCA CCT TAT TCT TGG (2813–2831)
P4: 5′ ATA ACT GAA AGC CAA ACA GTG GG (738–716)
Fragment 3: P5–P6
P5: 5′ GTC TTC TTG GTT GTT CTT CTA C (427–448)
P6: 5′ GCA GCA CAG CCT AGC AGC CAT GG (1394–1372)
Fragment 4: P7–P8
P7: 5′ CCA TAC TGC GGA ACT CCT AGC (1266–1286)
P8: 5′ CAA TGC TCA GGA GAC TCT AAG GC (2043–2021)

Plus and minus strands were sequenced with 16 dif-

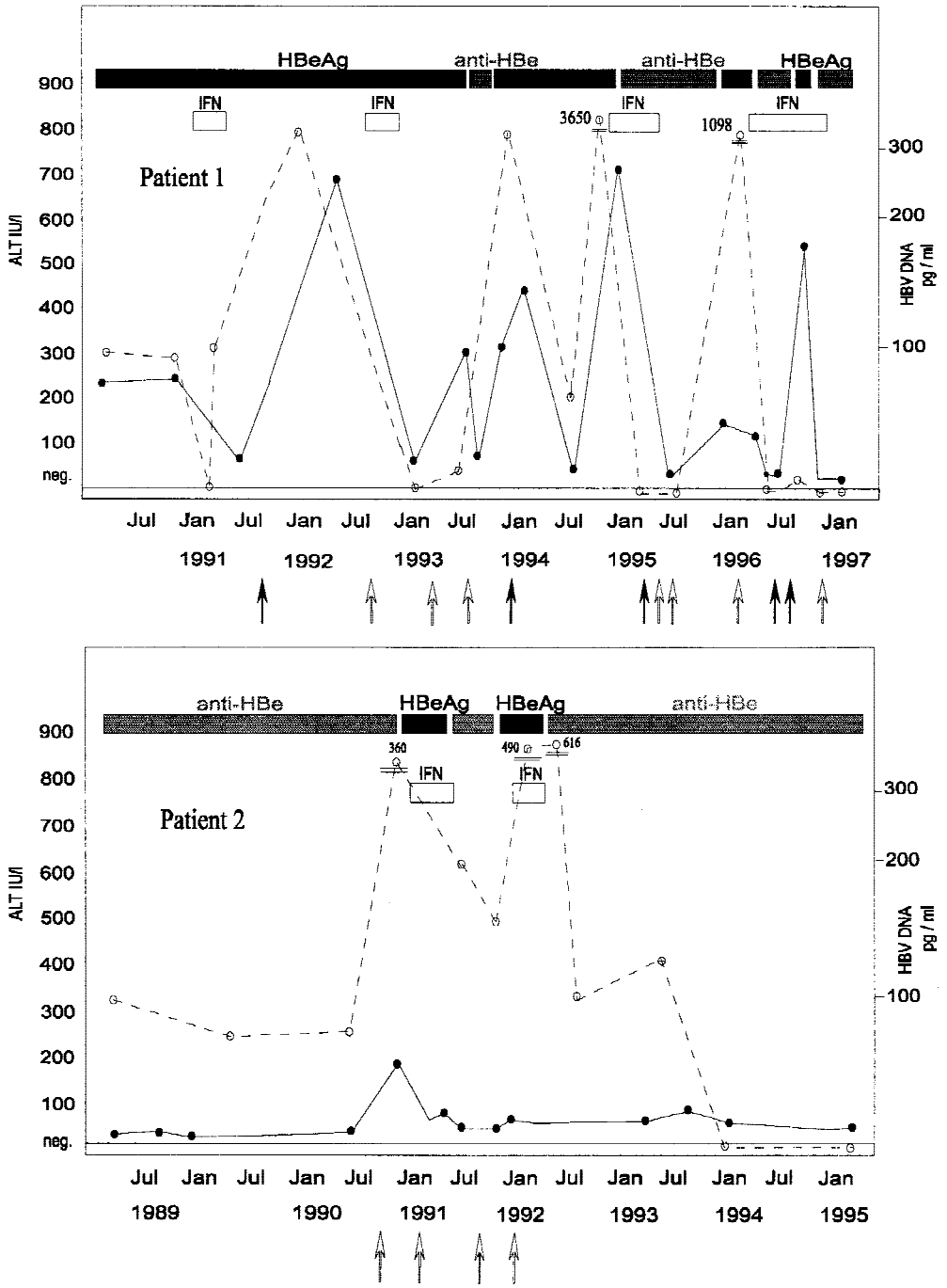

Fig. 1. Course of serological markers in patients 1 and 2. In patient 1, the periods of reactivations were preceded with marked elevations in serum alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA levels. In patient 2, no clear association between ALT and HBV DNA levels are seen. Arrows indicate the time in which serum samples were analyzed. Filled arrows: full-length genomic analysis of HBV genomes; open arrows: analysis of the basic core promoter and the preCore region. The open boxes indicate the period of alpha-interferon (IFN) therapy. The solid line denotes the biochemical course of ALT; the dashed line denotes the HBV DNA levels.

ferent sense and antisense primers using a Dye Terminator Cycle Sequencing Kit (ABI PRISM, Foster City, CA) in an automated sequencer model 377 (ABI).

Analysis of Core Promoter Activity

A PCR fragment including the BCP of mutant and wild-type ayw HBV [Galibert et al., 1979] was amplified using the primer pair P9: 5′ TGT CAA CGA CCG ACC TTG AG (1681–1700) and P8: 5′ CAA TGC TCA GGA GAC TCT AAG GC (2043–2021). Directional subcloning of the BCP region into the luciferase reporter gene vector pGL2basic (Promega) was done via the corresponding restriction sites *Mlu*I and *Bgl*II, respectively. The resulting constructs pGL-BCP and pGL-

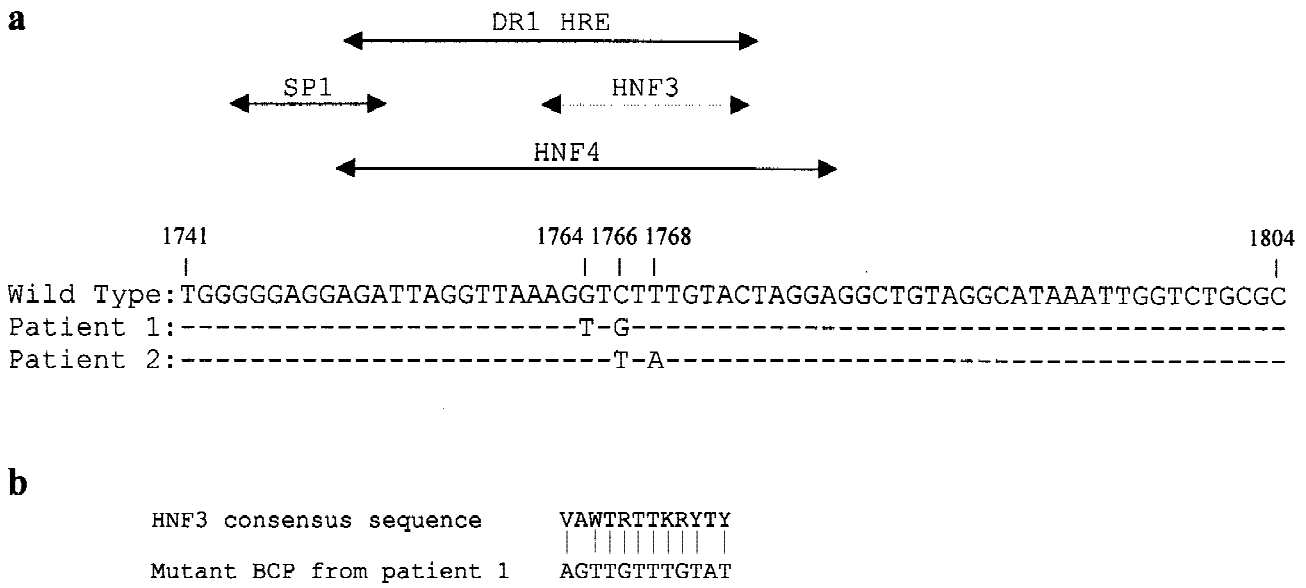

Fig. 2. **(a)** Mutations in the basic core promoter (BCP) from patients 1 and 2. The wild type sequence of genotype D [Bichko et al., 1985] is indicated at the top. The recognition sequences for hepatocyte nuclear factor 4 (HNF4), surface promoter 1 (SP1), and the direct repeat 1 hormone response element (DR1 HRE) are indicated by arrows. The DR1 HRE is bound by chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1), human testicular receptor 2 (TR2), peroxisome proliferator-activated receptors (PPARs), and retinoid X receptors (RXRs). The dashed arrow indicates the putative new binding site for hepatocyte nuclear factor 3 (HNF3) present in the mutated BCP of patient 1. **(b)** The putative binding site for HNF3 from patient 1 is aligned to the HNF3 consensus sequence. Mutated nucleotides are indicated and consensus nucleotides are shown as dashes.

ayw were sequenced and introduced in parallel, including pGL2basic, into THT-1 hepatoma cells [Harvey et al., 1997] maintained at conditions described. Transfections were undertaken in 10-cm dishes by the $CaPO_4$ method [O'Mahoney and Adams, 1994]. Transfection efficiency was monitored after cotransfection of the β-galactosidase expression plasmid pSVβ-Gal (Promega) by colorimetric or histochemical detection of enzyme activity [O'Mahoney and Adams, 1994]. Typically, more than 50% of cells were transfected. Luciferase activity was determined 48 hr after transfection using Promega's luciferase assay system and normalized of the amount of β-galactosidase activity. All plasmid constructs, including the pGL2basic vector as a control, were analyzed in triplicates. Promoter activity of mutant and wild-type HBV showed similar results in three independent experiments.

Identification and Quantification of Viral Subpopulations

According to Leitner et al. [1993], it is possible to detect and quantify roughly minor populations of sequence variants in as little as 10% of the total virus population. Peak areas and heights from directly sequenced PCR products were quantified by comparison of neighboring peaks of the same base (Fig. 3).

Analysis of HBV DNA in Liver Tissue

Liver biopsy from patient 1 was available 4 years before, and tumorous and nontumorous tissue was available immediately after diagnosis of HCC from a liver resection.

HBV DNA analysis was done by the Southern technique. After DNA extraction from the liver tissue by phenol/chloroform, 15 μg of DNA were digested by the restriction enzyme *Hind* III. The DNA fragments were electrophoresed in a 0.8% agarose gel and subsequently transferred to a nylon membrane (Nytran, Schleicher & Schuell). Molecular hybridization was carried out using an HBV DNA probe labeled with ^{32}P-CTP (Amersham) by nick translation to a specific activity of 2–5 × 10^8 cpm/μg of DNA. Autoradiography was done at −70°C with an intensifying screen (Hyperfilm-MP, Amersham). The time of exposure was 5 days.

Comparative Sequence Analysis

Using the sequence alignments available from the sequence database of the National Institute for Biotechnology Information (NCBI No: ID 1107590, 971267, 736003, 527440, 527435, 474959, 457780, 452641, 452637, 452632, 452622, 329667, 451966, 329649, 329633, 329628, 221499, 221498, 221497, 474959, 59585, 221494, 59416, 59402, 451966, 260397, 221499, 221497, 221494, 1359675, 329637, 452613, 221505, 59404, 59418, 59429, 1359690, 1359698, 1359682, 1359675, 1107583, 1107576, 313780, 1514493) and Norder et al. [1994], the HBV strains of patient 1 and 2 were classified as genotype D, subtype ayw2 (Genbank accession numbers: AF 151735 and AF 151736). Sequence alignments were performed using the Sequencher 3.0 software program.

RESULTS

Comparison of the HBV Genomes From Different Stages of the Disease

Serum samples from patient 1 obtained from 12 different stages of the disease were analyzed. Full-length

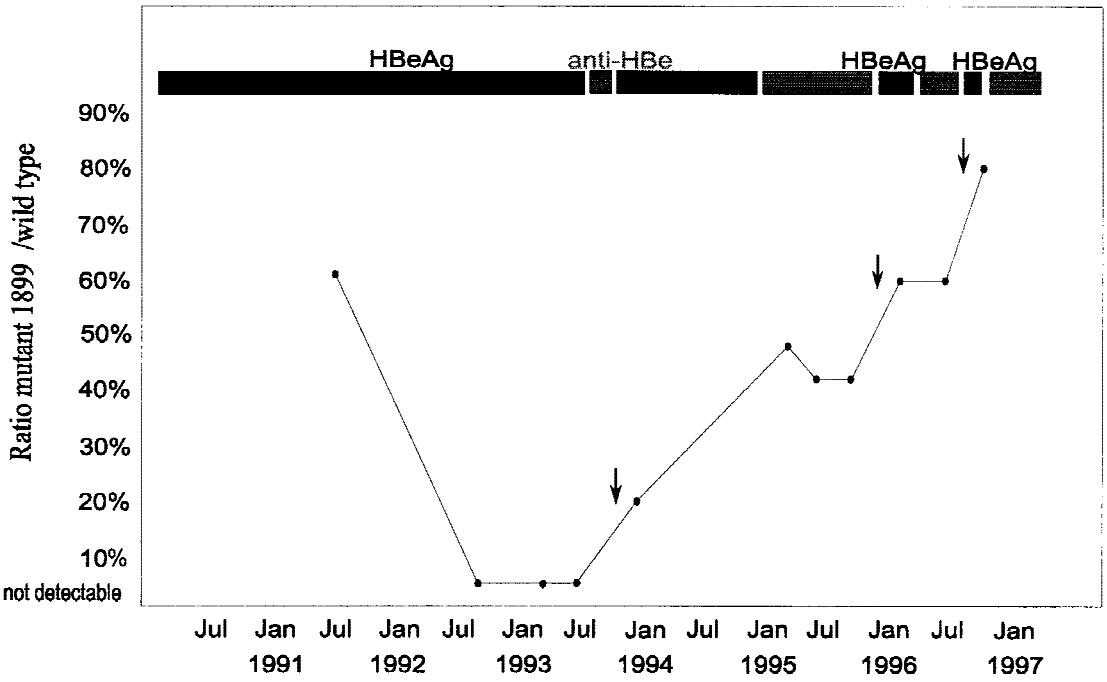

Fig. 3. Quantitative amounts of hepatitis B virus (HBV) mutant in relation to the wild-type genomes of the preCore region at nucleotide position 1899. Filled circles denote the times in which sera were analyzed. Arrows indicate the time of reactivations. Each reactivation from anti-hepatitis B e (anti-HBe) to hepatitis B e antigen (HBeAg) is accompanied by an increase of the population of 1899_A variant.

genomic analysis was carried out from five sera and the basic core promoter and preCore region were sequenced from seven additional HBV isolates. After amplification, the PCR products were sequenced directly from all but one sample, from which the entire HBV DNA sequences of four clones were sequenced.

Notably in patient 1, only 1 nucleotide at position $1899_{G\rightarrow A}$ was identified to vary among the viral genomes derived from the 12 HBV isolates. This mutation in the preCore region was found to coexist with the wild-type sequence either as minor or major population. The time of reactivation was always accompanied by an increase of the mutant 1899_A particles as shown in Fig. 3.

Four different sera were obtained from patient 2 and the BCP region was sequenced directly. The times in which serum samples were analyzed are shown in Figure 1. In patient 2, sequential changes between the several isolates were not observed.

Alignment of the HBV Genomes to the Reference Sequences

The HBV DNA sequences from patient 1 and 2 were aligned to 44 published complete HBV genomes and were classified as genotype D and subtype ayw2. In patient 1, the sequence alignment with the reference sequence [Bichko et al., 1985] revealed 53 nucleotide substitutions and additional 8 changes in the four subcloned viral populations. Nine of these changes were unique to the HBV genomes compared (Fig. 4 and Table I). As shown in Figure 4b, the identified mutations were distributed over the whole genome and no specific cluster of mutations was observed. The nucleotide exchanges predicted 37 amino acid changes in all viral proteins (Fig. 4a and Table II).

Due to the transactivating function of the HBx protein and the development of HCC in child 1, the predicted amino acid sequence should be noted. The HBx alterations in codons 46, 88, 102, and 131 were present in many other reference genomes, whereas a change in codon 33 was found rarely. This serine-to-proline substitution results in a drastic structural change of the peptide chain. However, changes in the amino terminus to position 45 have been shown to have only little or no effect on its protein activity [Runkel et al., 1993].

In both children, two base pairs differed from the wild-type sequence in the BCP (Fig. 2). The mutations at position $1764_{G\rightarrow T}$, $1766_{C\rightarrow G}$ in subject 1 and $1766_{C\rightarrow T}$, $1768_{T\rightarrow A}$ in case 2 were either unique or found rarely in the 44 references. The consensus sequence for the transcription factor binding site of hepatocyte nuclear factor 3 (HNF3) is shown in Figure 2b. The double mutation of patient 2 was isolated recently in an HBV variant associated with an outbreak of fulminant hepatitis [Baumert et al., 1996].

Biological Function and Significance of BCP Mutants

Analyses of the BCP activity were carried out by the luciferase assay. As shown in Figure 5, the mutant construct showed a 2.8-fold increase in the expression of the reporter gene.

The functional properties of the basic core mutations of patient 2 have been investigated recently. The two adjacent mutations at position 1766_T and 1768_A in-

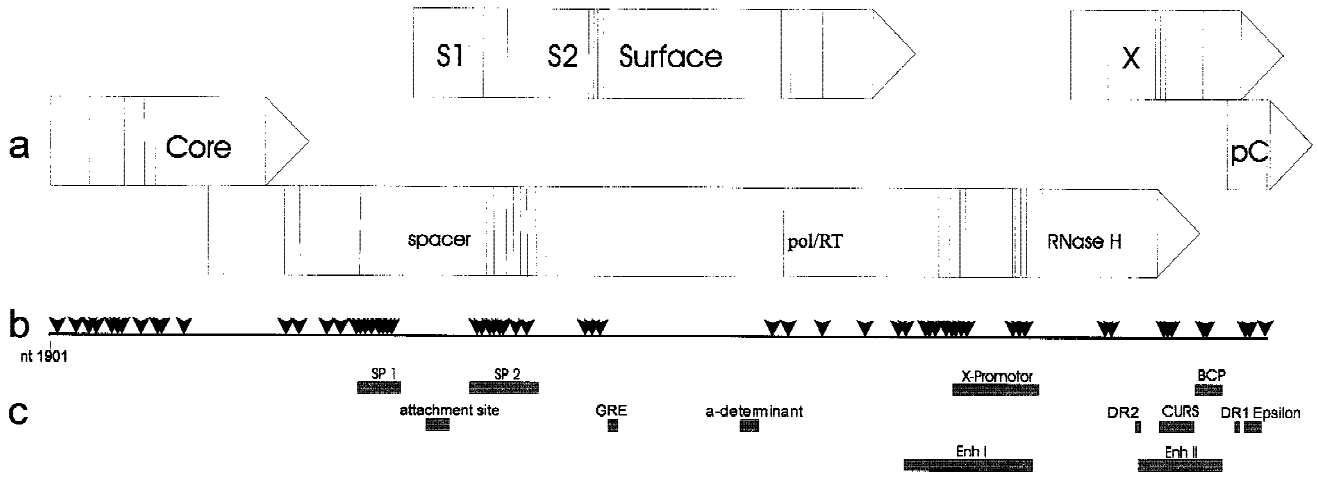

Fig. 4. **(a)** Location of the amino acid substitutions identified in patient 1. The four open reading frames of the hepatitis B virus (HBV) genome are shown as rectangles. Perpendicular lines indicate the differences to the reference sequence [Bichko et al., 1985]. Abbreviations in the open reading frames are as follows: S1, preS1; S2, preS2; x, xgen; pC, preCore; pol/RT, polymerase/reverse transcriptase; RNase H, ribonuclease H. **(b)** Location of nucleotide exchanges from patient 1. Arrows indicate the differences to the reference sequence. **(c)** Regulatory elements of the HBV genome. SP1, surface promoter 1; SP2, surface promoter 2; BCP, basic core promoter; GRE, glucocorticoid responsive element; DR1, direct repeat 1; DR2, direct repeat 2; Epsilon, epsilon encapsidation signal; CURS, core upstream regulating sequences; Enh I, enhancer I; Enh II, enhancer II.

TABLE I. Nucleotide Exchanges From Patient 1 in Comparison to the Reference Sequence

Nt position					
120	C→T	1470	T→C	2512	T→C*
132	G→A	1625	A→C	2554	A→G*
144	C→T	1631	A→T	2624	A→G
586	C→A	1633	C→A	2642	A→T
617	T→C*	1764	G→T	2705	T→C
720	C→T	1766	C→G	2712	T→C
815	C→T	1843	C→T	2715	T→C
926	T→G	1846	A→T	2738	T→C
938	C→T	1899	G→A	2768	G→A
961	G→A	1912	C→T	2772	A→G
967	A→C	1969	C→T	2780	C→T
973	A→G	1998	A→C	2792	T→A
1057	C→T*	2007	C→A*	3024	A→G
1059	T→C	2062	A→T	3031	G→A*
1080	G→T	2077	A→T	3070	G→C
1095	T→G*	2092	A→C	3072	A→C
1221	G→A	2139	C→T	3075	G→C
1250	T→C	2177	A→G	3076	T→G
1256	G→C	2188	A→G	3129	T→G
1258	C→G	2266	A→C	3159	T→G
1449	C→T*				

Shown are nucleotide exchanges from patient 1 which differ from the reference sequence [Bichko et al., 1985]. Shaded nucleotides indicate exchanges which are not present in the 44 previously published hepatitis B virus sequences. *Nucleotide substitutions only present in some clones.

TABLE II. Amino Acid Exchanges From Patient 1 in Comparison to the Reference Sequence

PreCore		Pol	
29	Gly→Asp	69	Val→Ala*
Core		83	His→Arg*
33	Asn→Thr	136	Tyr→His
64	Glu→Asp	240	Thr→Ala
80	Ala→Val	242	Arg→Lys*
93	Met→Val	255	Arg→Thr
PreS1		256	Asn→His
61	Gly→Ser*	257	Val→Arg
75	Gln→His	275	Tyr→Asp
PreS2		285	Ser→Ala
44	Ser→Leu	498	Ile→Thr
48	Arg→Lys	601	Ile→Arg
52	Pro→Leu	613	Val→Ile
Surface		617	Ile→Arg
144	Asp→Glu	645	Pro→Ser*
155	Ser→Pro*	699	Met→Ile
189	Thr→Ile	709	Leu→Pro
X		711	Arg→Pro
33	Ser→Pro	712	Leu→Val
82	Lys→Asp		
84	Gln→His*		
85	Pro→Gln		
129	Val→Leu		

Shown are amino acid exchanges from patient 1 that differ from the reference sequence [Bichko et al., 1985]. Shaded amino acids indicate exchanges that are not present in the 44 previously published hepatitis B virus (HBV) sequences. *Amino acid substitutions only present in some HBV clones.

creased the expression of the luciferase reporter gene by a factor of 2.3 [Baumert et al., 1996].

HCC and Viral Integration

Liver tissue from patient 1 was studied for viral integration sites 4 years before and immediately after diagnosis of HCC. As shown in Figure 6, only free viral sequences were detected in the liver biopsy before development of HCC (lane 1), whereas single site integration of viral DNA was present in both tumor and non-tumorous liver tissue after HCC development (lanes 2 and 3).

DISCUSSION

The functional significance of a double mutation within the BCP and whether specific viral variants

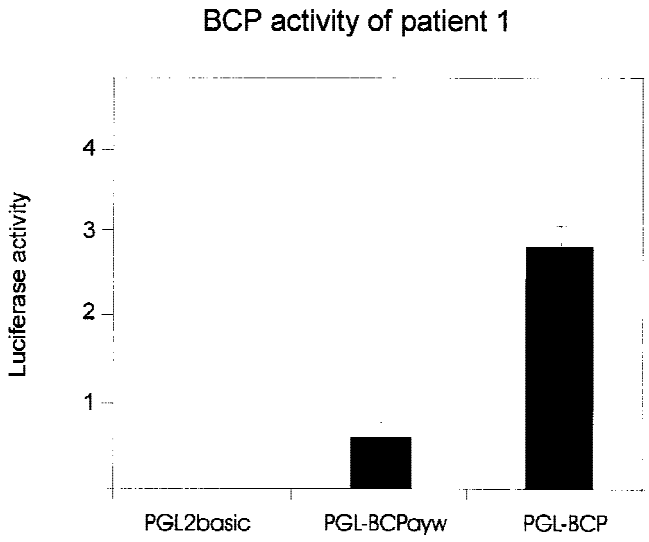

Fig. 5. Effect of mutant $1764_T/1766_G$ on the core promoter activity. Constructs containing no (pGL2basic), ayw core promoter (pGL2-ayw), and $1764_T/1766_G$ (pGL-BCP) core promoter are indicated at the *x*-axis. The data are the results of three independent experiments and demonstrate a 2.8-fold increase in core promotor activity of the mutant promotor from patient 1 in relation to the wild-type promotor.

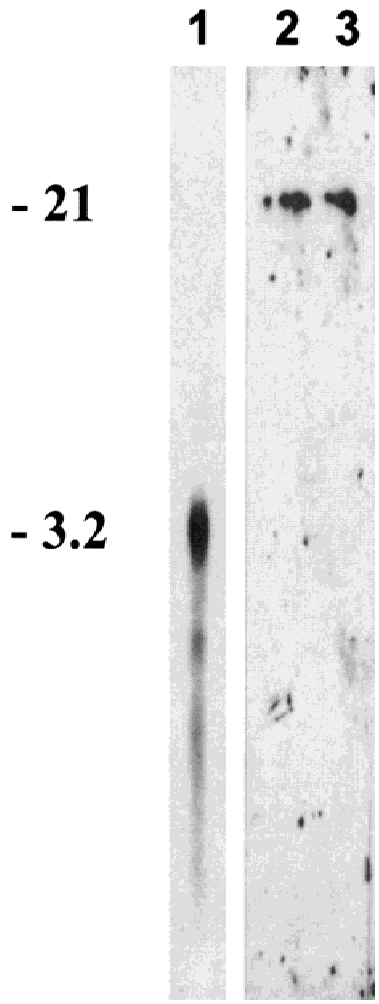

Fig. 6. Southern blot hybridization analysis of liver-biopsy tissue from patient 1 before (lane 1) and from liver resection material after the development of hepatocellular carcinoma (lanes 2 and 3). Lane 1 shows free hepatitis B virus (HBV) DNA with a single band at 3.2 kb and a smear below, indicating replicative intermediates (time of exposition 5 days). Lane 2 with nontumorous liver tissue and lane 3 with carcinoma cells show a single site integration of only integrated HBV DNA at approximately 21 kb (time of exposure 5 days). From each sample 15 μg of tissue was used, digested with *Hind* III, subjected to 0.8% agarose gel electrophoresis, and blotted on nitrocellulose paper.

may contribute to the exacerbations of HBV infection were investigated in two children with multiple viral reactivations. The role of HBV integration into the host genome in the development of HCC was also studied.

Twelve and 4 sera obtained before and after the viral flare-ups and the reconversions from anti-HBe to HBeAg were analyzed in patients 1 and 2, respectively. Both patients harbored mutations in the BCP, which is described as a region bound by numerous transcription factors (see Fig. 4) [Günther et al., 1996; Buckwold et al., 1997; Raney et al., 1997; Yu and Mertz, 1997]. Previous studies demonstrated that a double mutation at nucleotide position $1762_{A\rightarrow T}/1764_{G\rightarrow A}$ is detected frequently in chronic HBsAg carriers. Patients 1 and 2 in the present report also harbored double mutations in this region, at nt $1764_{G\rightarrow T}/1766_{C\rightarrow G}$ and nt $1766_{C\rightarrow T}/1768_{T\rightarrow A}$. However, the base exchanges were either unique or rarely present in comparison to 44 HBV genomes that were sequenced previously.

Recently, the functional consequences of the adjacent mutations at positions 1762/1764 and 1766/1768 have been shown to be associated with enhanced activity of the core promoter, increased core protein expression, decreased HBeAg levels, and a more replication-competent phenotype [Baumert et al., 1996, 1998; Buckwold et al., 1996; Moriyama, 1997]. Thus, there is strong evidence that variations in this part of the promoter alter the binding of transcription proteins to the HBV DNA. The finding that the region that is bound by so many proteins is involved in the highest frequency of mutations and sequence rearrangements in the core promoter, suggests that at least some of the biological effects are due to altered binding of protein factors. Günther et al. [1996] also identified the generation of novel HNF1 binding sites in immunosuppressed patients that affected expression and replication in a similar way as mentioned above. Although knowledge about the significance of BCP mutants is still limited, the finding that all mutations analyzed to date that enhanced the core promoter activity coincided with higher replication capacities of HBV variants, suggests that this effect is dependent on the level of promoter transformation. This effect is not unexpected, as the core promoter not only serves for translation of the core and pol proteins but is also used as the template for reverse transcription. In that way the core promoter regulates the replication of the virus. Hence, it appears likely that the HBV variant identified in patient 1 replicates more efficiently and thus may have an advantage in escaping immune clearance.

So far, explanations for HBV reactivations have only

been sought in the preCore/core ORF. Several studies have found the emergence of preCore stop codon mutants, particularly at nt 1896_A after the time of reactivation [Raimondo et al., 1990; Bortolotti et al., 1993]. However, many more cases are reported where preC stop mutants were observed even before reactivation or where reactivation was not accompanied by HBeAg-defective variants [Takeda et al., 1990; Loriot et al., 1995]. These reports are in agreement with our findings in patient 1 in whom HBeAg-minus mutants were not observed in any of the 12 sera.

In view of the above results, it is possible that HBV reactivation might have been triggered by another preC mutation at nt 1899_A. Remarkably it was the only genetic change in the HBV genome before and after reactivations. This mutation is found in a large number of chronically infected patients and is generally identified in coexistence with the 1896_A preC stop codon mutant, whereas the isolated mutant 1899_A is described rarely in association with exacerbation of the disease [Günther et al., 1992; Laskus et al., 1994; Carman et al., 1995; Sterneck et al., 1997]. As shown in Figure 3, it was striking that each viral reactivation of patient 1 was accompanied by an increase of the mutant 1899_A population. It has been suggested that the biological significance of the 1899 mutation may be related to the so-called "Kozak-sequence" around ATG start codons [Kozak, 1986; Carman et al., 1989]. In this motif, single base substitutions were identified to alter ribosomal binding and may thus enhance translation in eukaryotic cells. However, this hypothesis has never been demonstrated in HBV.

It was surprising that despite four anti-HBe-positive phases with a total duration of 24 months, only one mutation (position 1899_A) emerged during the chronic course of the disease. In a recent longitudinal study including 22 cases, it was shown that all patients displayed silent and missense mutations already 1–3 months after HBeAg/anti-HBe seroconversion [Gerner et al., 1998]. The stability of the HBV genomes from our two patients after two and four courses of treatment with IFN, respectively, is even more surprising because it was demonstrated that IFN exerts immunological pressure on the virus resulting in the selection and emergence of HBV variants [Günther et al., 1992].

Another interesting finding of this study was the identification of integrated viral DNA immediately after the development of HCC. Because only cellular HBV DNA was found before carcinogenesis began in the liver of the 8-year-old patient, it may be that integration of HBV DNA into the host's cellular genome hit a critical region and induced tumorigenesis either by activation of an oncogene by insertion of a promoter or enhancer of HBV next to it or by loss of anti-oncogene function.

In conclusion, patients with multiple anti-HBe reactivations were found to display mutations in the BCP leading to enhanced core promoter activity. This finding could be a result of novel or altered binding sites for liver-enriched and ubiquitous transcription factors. The fact that these variants were detected rarely in chronically infected patients with mild liver disease suggests that they may play a role in the pathogenesis of HBV reactivation.

ACKNOWLEDGMENTS

We thank E. Graef for technical support. We also thank S. Günther for helpful discussions. This work was carried out at the University of Mainz, Germany.

REFERENCES

Baumert TF, Marrone A, Vergalla J, Liang TJ. 1998. Naturally occurring mutations define a novel function of the hepatitis B virus core promoter in core protein expression. J Virol 72:6785–6795.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ. 1996. Two core promoter mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest 98:2268–2276.

Bichko V, Pushko P, Dreilina D, Pumpen P, Gren E. 1985. Human hepatitis B virus strain ayw variant. FEBS Lett 185:208–212.

Bortolotti F, Crivellaro C, Brunetto MR, Cadrobbi P, Bertolini A, Alberti A. 1993. Selection of a precore mutant of hepatitis B virus and reactivation of chronic hepatitis B acquired in childhood. J Pediatr 123:583–585.

Buckwold E, Xu Z, Chen M, Yen BTS, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 70:5845–5851.

Buckwold VE, Xu Z, Yen TS, Ou JH. 1997. Effects of a frequent double nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication. J Gen Virol 78:2055–2065.

Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 9:588–591.

Carman WF, Korula J, Wallace L, McPhee R, Mimms L, Decker R. 1995. Fulminant reactivation of hepatitis B due to an envelope protein mutant that escaped detection by monoclonal HbsAg ELISA. Lancet 345:1406–1407.

Friedt M, Gerner P, Lausch E, Trübel H, Zabel B, Wirth S. 1999. Mutations in the core promoter and the precore region of hepatitis B virus and their selection in children with fulminant and chronic hepatitis B. Hepatology 29:1252–1258.

Galibert FE, Mandart E, Fitoussi F, Tiollais P, Charnay P. 1979. Nucleotide sequences of the hepatitis B virus genome subtype ayw cloned in *E. coli.* Nature 281:646–650.

Gerner PR, Friedt M, Oettinger R, Lausch E, Wirth S. 1998. The hepatitis B virus seroconversion to anti-HBe is frequently associated with HBV genotype changes and selection of preS2 defective particles in chronically infected children. Virology 245:163–172.

Günther S, Meisel H, Reip A, Miska S, Krüger DH, Will H. 1992. Frequent and rapid emergence of mutated pre-C sequences in HBV from e-antigen positive carriers who seroconvert to anti-HBe during interferon treatment. Virology 187:271–279.

Günther S, Piwon N, Iwanska A, Schilling R, Meisel H, Will H. 1996. Type, prevalence and significance of core promoter/enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. J Virol 70:8318–8331.

Harvey T-J, MacNaughton TB, Gowans EJ. 1997. The development and characterisation of a SV40 T-antigen positive cell line of human hepatic origin. J Virol Methods 65:67–74.

Hasegawa K, Huang J, Rogers SA, Blum HE, Liang TJ. 1994. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. J Virol 68:1651–1659.

Inoue K, Yoshiba M. Sekiyama K, Okamoto H, Mayumi M. 1998. Clinical and molecular virological differences between fulminant

hepatic failures following acute and chronic infection with hepatitis B virus. J Med Virol 55:35–41.

Kozak M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 31:283–292.

Kurosaki M, Enomoto N, Asahina Y, Sakuma I, Ikeda T, Tozuka S, Izumi N, Sato C. 1996. Mutations in the core promoter region of hepatitis B virus in patients with chronic hepatitis B. J Med Virol 49:115–123.

Laskus T, Rakela J, Tong MJ, Nowicki MJ, Mosley JW, Persing DH. 1994. Naturally occurring hepatitis B virus mutants with deletions in the core promoter region. J Hepatol 20:837–841.

Leitner T, Halapi E, Scarlatti G, Rossi P, Albert J, Fenyö EM, Uhlen H. 1993. Analysis of heterogeneous viral populations by direct DNA sequencing. Biotechniques 15:120–127.

Lindh M, Gustavson C, Mardberg K, Norkrans G, Dhillon AP, Horal P. 1998. Mutation of nucleotide 1762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. J Med Virol 55:185–190.

Loriot MA, Marcellin P, Talbodec N, Guigonis V, Gigou M, Boyer N, Bezeaud A, Erlinger S, Benhamou J-P. 1995. Low frequency of precore Hepatitis B virus mutants in anti-hepatitis B e-positive reactivation after loss of hepatitis B e antigen in patients with chronic hepatitis B. Hepatology 21:627–631.

Moriyama K. 1997. Reduced antigen production by hepatitis B virus harbouring nucleotide deletions in the overlapping X gene and precore-core promoter. J Gen Virol 78:1479–1486.

Norder H, Courouce A-M, Magnius LO. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198:489–503.

O'Mahoney JV, Adams TE. 1994. Optimization of experimental variables influencing reporter gene expression in hepatoma cells following calcium phosphate transfection. DNA Cell Biol 12:1227–1232.

Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 68:8102–8110.

Raimondo G, Stemler M, Schneider R, Wildner G, Squadrito G, Will H. 1990. Latency and reactivation of a precore mutant hepatitis B virus in a chronically infected patient. J Hepatol 11:374–380.

Raney AK, Johnson JL, Palmer CA, McLachlan A. 1997) Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J Virol 71:1058–1071.

Runkel L, Fisher M, Schaller H. 1993. Two-codon insertion mutations of the HBx define two separate regions necessary for its transactivation function. Virology 197:529–536.

Sterneck M, Günther S, Gerlach J, Naoumov NV, Santantonio T, Fischer L, Rogiers X, Greten H, Williams R, Will H. 1997. Hepatitis B virus sequence evolving in liver transplant recipients with fulminant hepatitis. J Hepatol 26:754–764.

Sterneck M, Kalinina T, Otto S, Günther S, Fischer L, Burdelski M, Greten H, Broelsch CE, Will H. 1998. Neonatal fulminant hepatitis B: structural and functional analysis of complete hepatitis B virus genomes from mother and infant. J Infect Dis 177:1378–1381.

Takeda K, Akahane Y, Suzuki H, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M. 1990. Defects in the precore region of the genome in patients with chronic hepatitis B after sustained seroconversion from HBeAg to anti-HBe induced spontaneously or with interferon therapy. Hepatology 12:1284–1289.

Yu X, Mertz J. 1997. Differential regulation of the pre-C and pregenomic promoters of human hepatitis B virus by members of the nuclear receptor superfamily. J Virol 71:9366–9374.